

EXTENDED REPORTS

Increased cellularity and expression of adhesion molecules in muscle biopsy specimens from patients with rheumatoid arthritis with clinical suspicion of vasculitis, but negative routine histology

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Abstract

Objective—Histological analysis of random quadriceps muscle biopsy specimens can be used to detect vasculitis in patients with rheumatoid arthritis (RA). This study aimed at determining the immunohistological features in patients with clinical suspicion of rheumatoid vasculitis, but without a transmural infiltrate or fibrinoid necrosis of the vessel wall on routine histology.

Methods—Three groups of patients with RA were studied: (a) without clinical signs of vasculitis (n=6); (b) with recent onset of extra-articular features and a clinical suspicion of vasculitis but normal routine histology (n=11); and (c) with recent onset of extra-articular features and vasculitis, histologically proved either in muscle or other biopsy specimens (n=14). A control group of patients with osteoarthritis was also included (n=5). Frozen sections from quadriceps muscle biopsy specimens were analysed with monoclonal antibodies to detect CD3, CD4, CD8, CD68, ICAM-1, VCAM-1, and HLA-DR. The slides were evaluated using a semiquantitative scoring system (0–4).

Results—The mean scores gradually increased from group 1 to 3, leading to significant differences between groups 1 and 2, but not between groups 2 and 3 for most markers ($p < 0.05$). Thus the pathological changes were similar for the two groups with clinical signs of vasculitis, even when the conventional histological evaluation was negative. Higher immunohistological scores were associated with perivascular infiltrates on routine histology.

Conclusion—The pathophysiological events leading to vasculitis are reflected by the changes in the quadriceps muscle biopsy specimens. The data indicate that the sensitivity of examination of muscle biopsy specimens for the diagnosis of

rheumatoid vasculitis can be increased by the use of new criteria.

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In patients with rheumatoid arthritis (RA), vasculitis is a complication with several clinical presentations, which can be sometimes difficult to diagnose.^{1–3} Inflammation of small and medium sized vessels can cause skin disorders such as rash, cutaneous ulcerations and gangrene, neuropathy, eye symptoms, and may also affect visceral organs.

The clinical picture is of help in the diagnostic process, but most clinicians rely on histology for the diagnosis.^{3–4} Biopsy specimens can be obtained from clinically affected organs whenever these are accessible—for instance, in cases of peripheral neuropathy,⁵ but often randomly obtained biopsy specimens of skeletal muscle are used as an alternative.^{6–8} Other possibilities include rectum or labial salivary gland biopsy specimens.^{9–10} The histological gold standard for the diagnosis of vasculitis is the presence of a transmural infiltrate or fibrinoid necrosis of the vessel wall.^{11–12} In skin biopsy specimens leucocytoclasia is used as a diagnostic criterion.

The sensitivity for the diagnosis vasculitis is limited (<50%) when randomly obtained muscle biopsy specimens are examined¹³ owing to the segmental distribution of the histological abnormalities and, usually, the restricted organ involvement.¹² The diagnostic yield of muscle samples can be improved by examining multiple biopsy sections and by using new diagnostic criteria for vasculitis, such as the presence of perivascular infiltrates of more than three cell layers. This criterion is more sensitive and equally specific for the diagnosis of rheumatoid vasculitis, when compared with the criteria described above.⁸

In addition, immunohistological techniques might offer diagnostic possibilities beyond conventional morphological examination by quantification of certain functional aspects of the structures involved.

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Table 1 Characteristics of the individual patients with rheumatoid arthritis (RA) and extra-articular manifestations, including the sites at which biopsy samples were taken and whether vasculitis was present (+) or absent (-) in these specific histological samples

Patients*	Extra-articular manifestations	Quadriceps	Tibialis muscle	Skin	Suralis
RV 1	Purpura, nailfolds, leg ulcer, weight loss	+	ND	+	ND
RV 2	Purpura, episcleritis, DSSN*	+	ND	ND	ND
RV 3	Nailfolds, weight loss	+	ND	ND	ND
RV 4	Purpura, nailfolds	+	-	+	ND
RV 5	Nailfolds, DSSN, weight loss	+	-	ND	ND
RV 6	Purpura	+	+	+	ND
RV 7	Purpura	+	-	+	ND
RV 8	Purpura	-	-	+	ND
RV 9	Nailfolds, episcleritis, leg ulcer, weight loss	-	-	+	ND
RV 10	DSSN, weight loss	-	-	ND	+
RV 11	Purpura	-	-	+	ND
RV 12	Episcleritis, weight loss	-	+	ND	ND
RV 13	Nailfolds, DSSN	-	+	ND	ND
RV 14	Purpura	-	-	+	ND
RA+ 1	DSSN	-	ND	ND	-
RA+ 2	Fibrosing alveolitis	-	ND	ND	ND
RA+ 3	Nailfolds, scleromalacia, leg ulcer, DSSN	-	-	-	-
RA+ 4	DSSN, fever, weight loss	-	-	ND	-
RA+ 5	Nailfolds, weight loss	-	-	ND	ND
RA+ 6	Leg ulcer, nodulosis	-	-	ND	ND
RA+ 7	Weight loss	-	-	ND	ND
RA+ 8	Nailfolds	-	-	ND	ND
RA+ 9	DSSN	-	-	ND	-
RA+ 10	Nailfolds, weight loss	-	-	ND	ND
RA+ 11	Leg ulcer, weight loss	-	-	ND	ND

*RV = patients with RA with extra-articular manifestations and histologically proved vasculitis (n=14); RA+ = patients with RA with extra-articular manifestations but no histological proof of vasculitis (n=11); DSSN = distal symmetrical sensory or sensorimotor neuropathy.

This study aimed at examining the cell infiltrate and the expression of adhesion molecules in muscle biopsy specimens from patients with rheumatoid vasculitis in order to determine whether immunohistological analysis may have an additional diagnostic value compared with routine histological examination.

Methods

PATIENTS

Three groups of patients with RA were selected. Firstly, patients without any clinical sign of vasculitis were included. In this so-called "RA-" group the mean age of the six patients with RA was 72 years, with a mean disease duration of 12 years. Rheumatoid factors were detectable in five out of six, all had erosive disease, and none had subcutaneous nodules. Four of the patients in this group were treated with disease modifying antirheumatic drugs (DMARDs) (three sulfasalazine, one methotrexate) and one patient used prednisone (13 mg/d).

Secondly, patients with recent onset of extra-articular features (<6 months) but no histological proof of vasculitis were included. This "RA+" group comprised 11 patients with RA with a mean age of 65 years and a mean disease duration of 19 years. All were rheumatoid factor positive and had erosive disease. A majority had subcutaneous nodules (6/11). Of the patients in this group eight of 11 used DMARDs (three sulfasalazine, three intramuscular gold, one methotrexate, and one azathioprine). Two patients used prednisone (10 mg/d). The mean swollen joint count in this group was 10.6 (SD 4.0) and the mean tender joint count 13.5 (SD 9.3).

Thirdly, patients with recent onset of extra-articular features and histological proof of vasculitis were included. This so-called rheuma-

toid vasculitis ("RV") group consisted of 14 patients with RA with a mean age of 71 years and a mean disease duration of 15 years.

All patients were rheumatoid factor positive, most of them had erosive disease (13/14), and a majority had subcutaneous nodules (9/14). In this group eight of 14 patients used DMARDs (six sulfasalazine and three hydroxychloroquine) and four of 14 used prednisone (three 10 mg/d, one 40 mg/d). In the subgroup of patients in which vasculitis was proved by quadriceps muscle biopsy (n=7), the mean swollen joint count was 7.4 (SD 4.4) and the tender joint count 8.7 (SD 6.7). In the subgroup of patients (n=7) with histological confirmation of vasculitis in other biopsy specimens, the mean swollen joint count was 10 (SD 2.5) and the mean tender joint count 9.3 (SD 3.0).

The extra-articular manifestations of RA were mainly nailfold lesions, purpura, cutaneous ulcers, and distal sensorimotor or sensory polyneuropathy. Weight loss was a common extra-articular feature, but it was almost always accompanied by other extra-articular manifestations of RA. Table 1 gives details of the extra-articular manifestations of the individual patients. In addition to the RA- control group, five patients with osteoarthritis (OA) with a mean age of 67 years were recruited.

HISTOLOGICAL ANALYSIS

Patients with RA were assigned to the different subgroups according to the results of an extensive conventional histological investigation on paraffin embedded tissue before, and independently of, the immunohistological analysis on frozen sections, which is the subject of this study.¹⁴ For this purpose a full thickness (skin/muscle/fascia) biopsy specimen of the quadriceps muscle was examined in all patients. Quadriceps muscle biopsy samples in the con-

Table 2 Infiltration by CD3+ T cells, CD4+ cells, CD8+ cells, CD68+ macrophages, and expression of the adhesion molecules ICAM-1 and VCAM-1 and the activation antigen HLA-DR in muscle biopsy specimens from patients with osteoarthritis (OA), rheumatoid arthritis (RA) without any signs of vasculitis (RA-), patients with RA with clinical signs of vasculitis without proof on routine histology (RA+), and patients with RA with histologically proved vasculitis (RV). Values are the mean semiquantitative scores (standard error)

	CD3	CD4	CD8	CD68	ICAM-1	VCAM-1	HLA
OA (n=5)	0.6 (0.2)	0.6 (0.2)	0.4 (0.2)	1 (0)	1 (0)	0.6 (0.2)	1 (0.6)
RA- (n=6)	0.5 (0.2)	0.7 (0.2)	0.2 (0.2)	1.2 (0.3)	1.2 (0.2)	0.2 (0.2)	1 (0.3)
RA+ (n=11)	1.7* (0.2)	1.5* (0.2)	1.3* (0.3)	2.3* (0.2)	2* (0.3)	1.4* (0.3)	2.6* (0.3)
RV (n=14)	2† (0.3)	2.1† (0.3)	1.8† (0.3)	2.6† (0.2)	2.7† (0.3)	1.9† (0.3)	2.8† (0.2)

*p<0.05 (Student's *t* test for differences between RA- and RA+).

†p<0.05 (Student's *t* test for differences between RA- and RV).

The difference between RA+ and RV was not statistically significant.

trol groups were obtained during surgery for total hip replacement or knee arthroplasty. In most of the patients with RA suspected of vasculitis an additional tibial muscle biopsy specimen was examined (table 1).

The presence of vasculitis was considered proved when fibrinoid necrosis of the vessel wall was shown by extensive histological examination after staining with haematoxylin and eosin, either in a quadriceps muscle or a tibialis muscle biopsy specimen.

In the absence of fibrinoid necrosis in the first series of sections, a total of 45 sections for each muscle biopsy sample was examined to allow optimal classification by avoiding false negative test results due to discontinuous distribution of the lesions. If no vasculitis was seen according to the conventional criteria in either muscle biopsy specimen, a sural nerve biopsy was done to confirm the diagnosis in patients with peripheral neuropathy, with suggestive electromyographic abnormalities of the sural nerve. Furthermore, a skin biopsy was performed in all patients with a skin rash to confirm or exclude the diagnosis of cutaneous vasculitis (table 1). All quadriceps muscle biopsy specimens were also evaluated for the presence of perivascular infiltrates, defined as the presence of at least three layers of mono/polymorphonuclear cells, around at least 50% of the circumference of a vessel wall.⁸

IMMUNOHISTOLOGICAL ANALYSIS

A part of each quadriceps muscle sample was snap frozen in Tissue-Tek OCT (Miles Inc-Diagnostic Division, Elkhart, IN) by immersion in methylbutane (-70°C). Only quadriceps muscle sections were used for the immunohistological investigations, even when by routine histology vasculitis was shown only in tibial muscle, skin, or sural nerve biopsy samples.

Frozen blocks were stored at -70°C until sectioned for immunohistological staining. Serial sections of 7 µm were cut on a cryostat and placed on glass slides (Star Frost adhesive slides, Knittelgläser, Germany).

Slides were fixed in acetone at room temperature for 10 minutes. Sections were stained with the following monoclonal antibodies: anti-CD3 (Becton-Dickinson, San Jose, CA), anti-CD4 (Becton Dickinson), anti-CD8 (Dako, Glostrup, Denmark), anti-CD68 (Dako), anti-ICAM-1 (MEM-111, Sanbio, Uden, The Netherlands), anti-VCAM-1 (1G11B1, Sanbio), and anti-HLA-DR (Dako).

After incubation with the primary antibody, affinity purified, horseradish peroxidase conjugated goat-antimouse (Dako) and swine-antigoat antibodies (Tago, Burlingame, CA) were used for detection of the cell markers and adhesion molecules according to the three step immunoperoxidase method, as described previously.¹⁵

In control sections the primary antibody was omitted or irrelevant antibody was applied at the same concentration as the primary antibody. Aminoethylcarbazole (Sigma, St Louis, MO) was used as dye in the immunoperoxidase reaction and hydrogen peroxide as substrate. Slides were counterstained with Mayer's haemalum (Merck, Darmstadt, Germany).

MICROSCOPIC EXAMINATION

After repeated evaluation of all sections, a specific set of standard sections was selected for each cell marker, corresponding to a semiquantitative score on a five point scale. A score of 0 represented minimum infiltration, while a score of 4 represented infiltration with numerous inflammatory cells. For VCAM-1 and HLA-DR, standard sections were chosen in a similar way. These standard sections were used as a gauge to score all other sections by comparison.^{16,17} All biopsy specimens were blinded and scored by two independent observers according to this validated semiquantitative scoring system.

Scores never differed by more than one point between observers. Whenever there was initial disagreement on a particular score a consensus score was determined.

STATISTICAL ANALYSIS

The mean scores for each marker were compared between the patient groups and statistical significance was evaluated with variance analysis and Student's *t* tests. A separate comparison between the RA+ and the RV group was made to check whether immunohistology might be of any value for differential diagnosis.

In addition, a subdivision between patients with or without perivascular infiltrates of more than three cell layers on routine histology was made in the RA+ and the RV group. The mean immunohistological scores of these subgroups were compared separately.

Results

After the routine histological examination seven patients were assigned to the RV group on the basis of a positive quadriceps biopsy, two were assigned to this group on the basis of

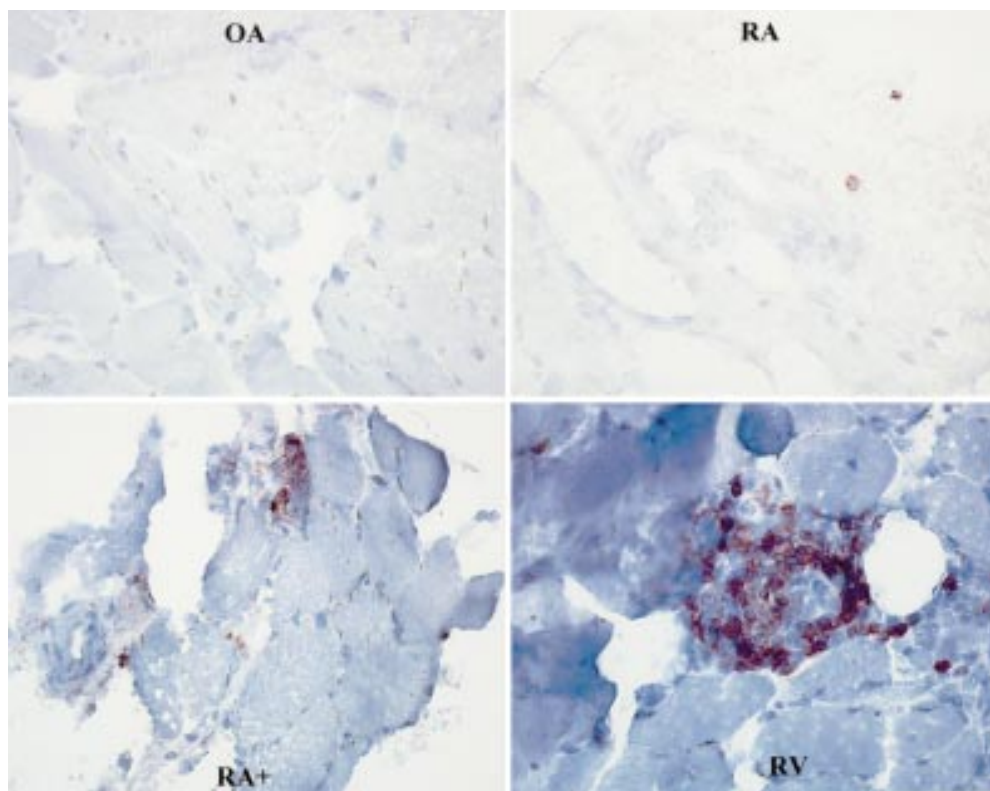


Figure 1A CD3+ T cells.

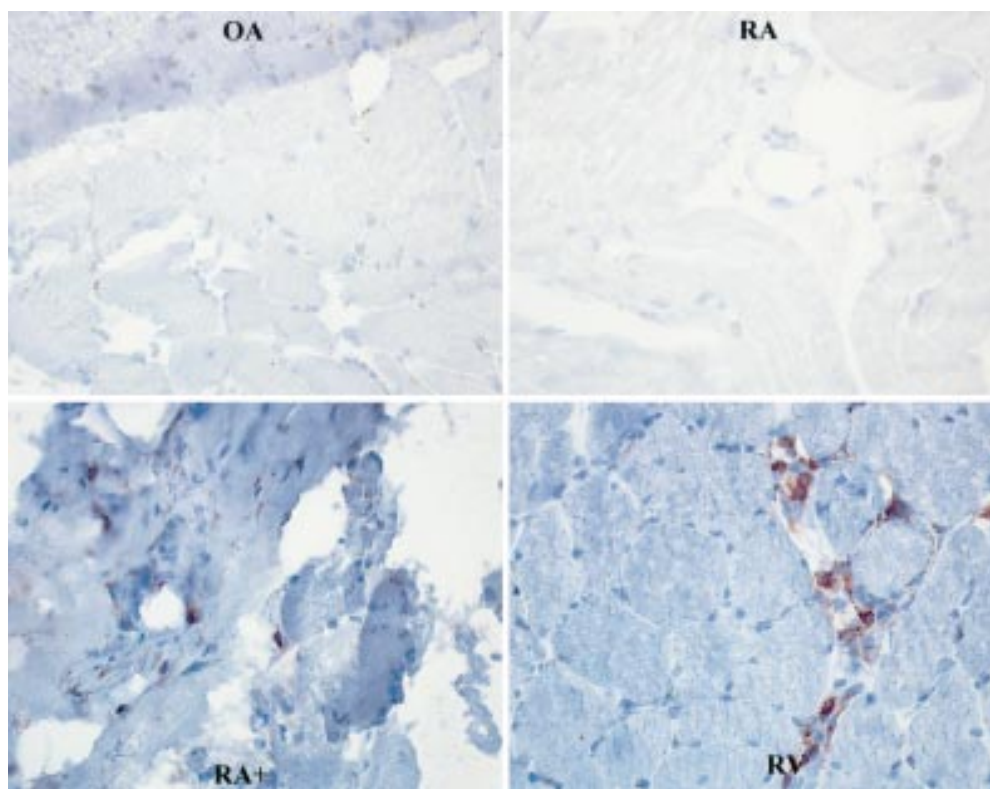


Figure 1B CD4+ cells.

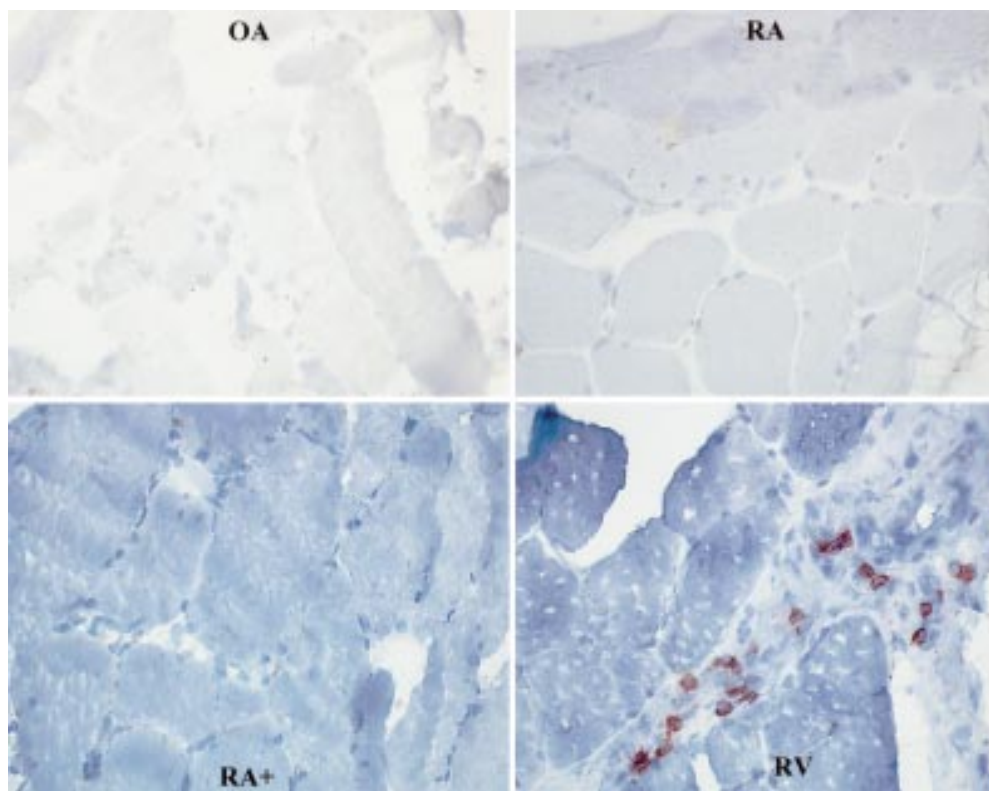


Figure 1C CD8+ cells.

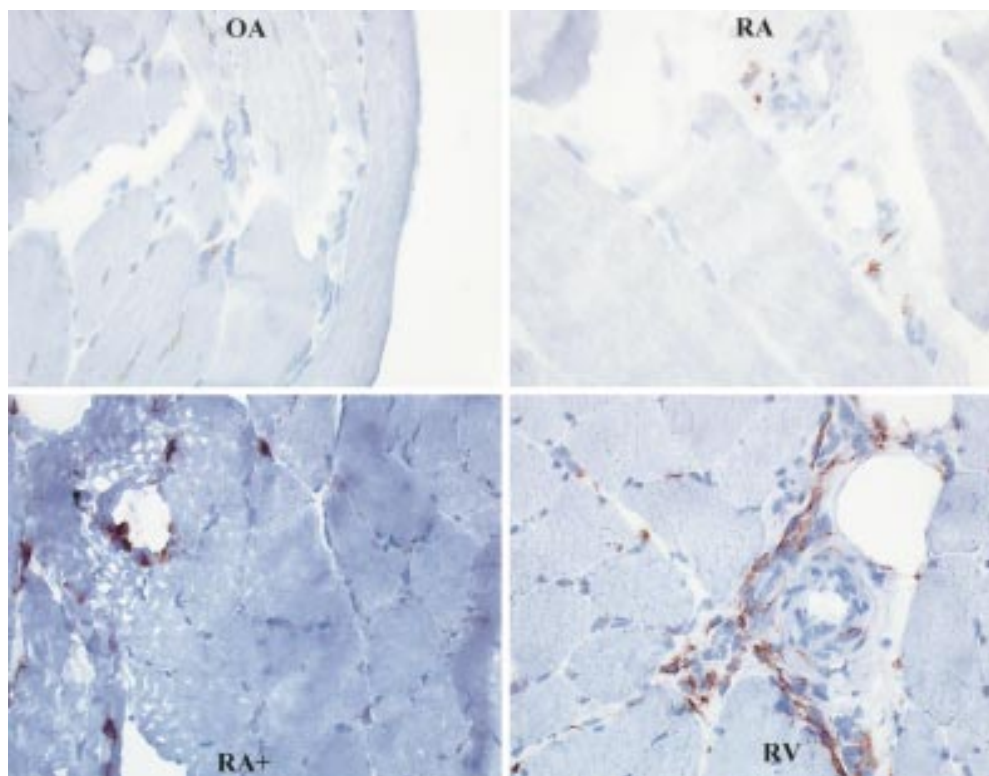


Figure 1D CD68+ macrophages.

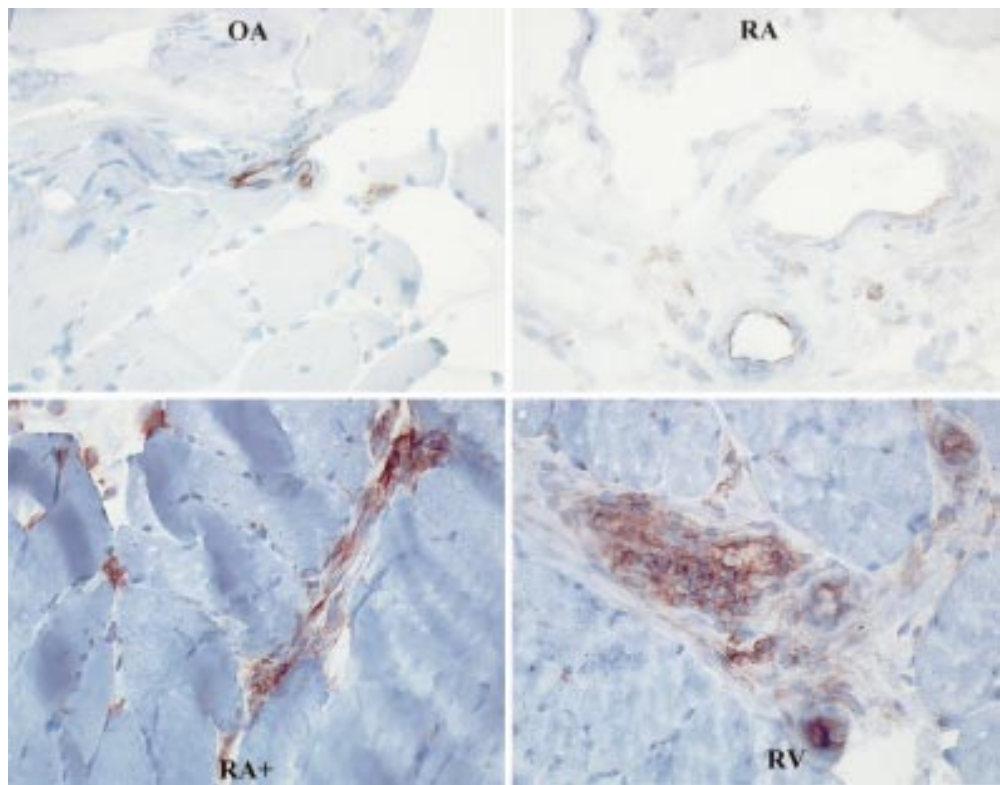


Figure 1E Adhesion molecule ICAM-1.

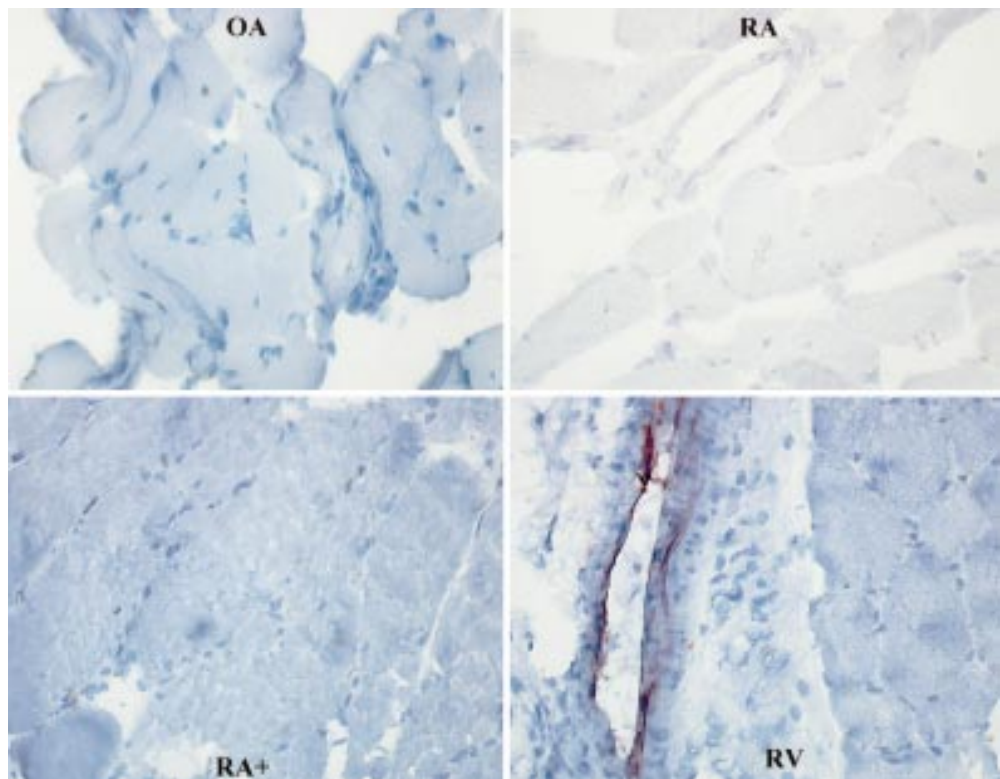


Figure 1F Adhesion molecule VCAM-1.

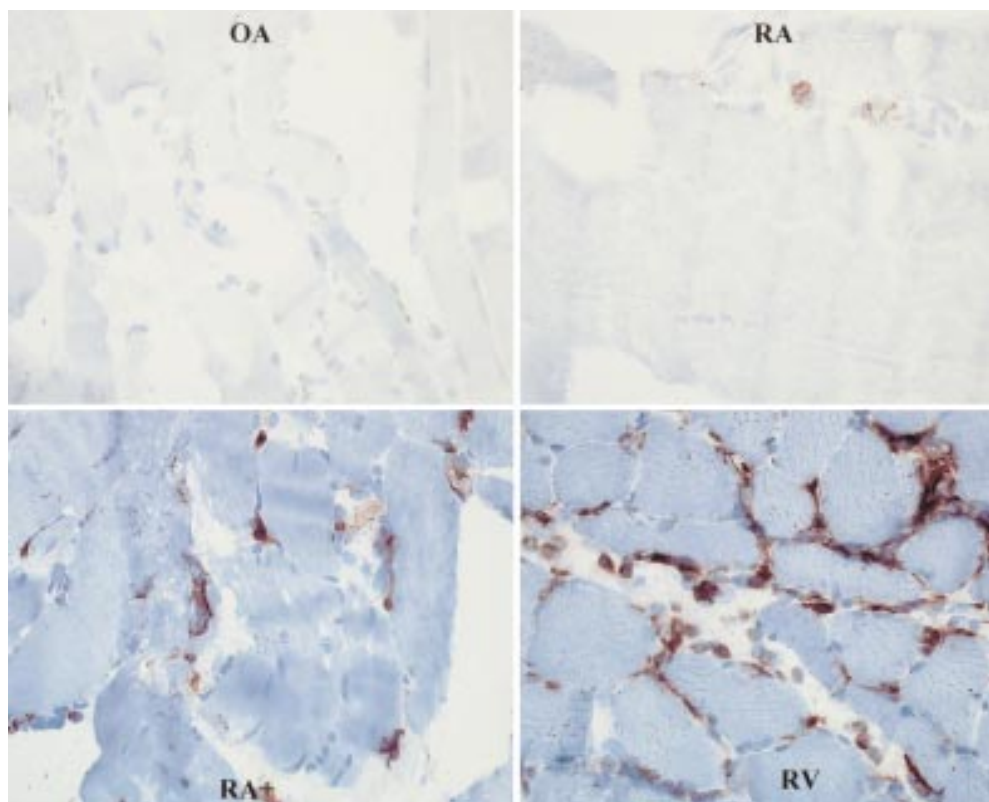


Figure 1G Activation marker HLA-DR.

Figure 1 Infiltration by (A) CD3+ T cells, (B) CD4+ cells, (C) CD8+ cells, and (D) CD68+ macrophages, and expression of adhesion molecules (E) ICAM-1 and (F) VCAM-1, and activation marker (G) HLA-DR in muscle biopsy specimens from patients with rheumatoid arthritis without clinical suspicion of vasculitis (group 1 = RA-), patients with rheumatoid arthritis with clinical evidence of vasculitis but without histological proof (group 2 = RA+), and patients with histologically proved rheumatoid vasculitis (group 3 = RV). The mean semiquantitative scores gradually increased from group 1 to group 3. Scores for the osteoarthritis (OA) control group were similar to the RA- group. (Original magnification $\times 400$.)

a positive musculus tibialis biopsy, one on the basis of a positive nervus suralis biopsy, and four on the basis of a positive skin biopsy. Vasculitis was found concomitantly in a quadriceps muscle biopsy specimen as well as in a skin biopsy sample in four cases, and in a quadriceps muscle biopsy specimen as well as in a musculus tibialis biopsy sample in one case (table 1). Patients assigned to the RA+ group did not show any sign of fibrinoid necrosis on

routine histology, either in the quadriceps muscle sample, which was taken in all of them, or in additional biopsy specimens. During the routine histological examination of the quadriceps muscle biopsy specimen perivascular infiltrates were documented in 11 of 14 patients in the RV group and in four of 11 patients in the RA+ group. No perivascular infiltrates were seen in the RA- and the OA group.

A few immunohistological slides could not be evaluated owing to the poor quality of the tissue. These included one ICAM-1 slide in each patient group, two CD8 slides in the RA+ and one in the RV group, and one CD4 slide in the RA+ group. All other sections were of good quality. A gradual increase in the mean immunohistological scores was recorded from group 1 (RA-), to group 2 (RA+), to group 3 (RV) (table 2). The scores in the RA- group were similar to those in the OA control group. Significantly higher mean scores were seen in the RA+ group for all markers than in the RA- group ($p < 0.05$). In the RV group all scores tended to be even higher, although the differences with the RA+ group did not reach statistical significance (fig 1).

Generally, patients with perivascular infiltrates on routine histology had significantly higher mean immunohistological scores for all markers than those without such infiltrates (fig 2).

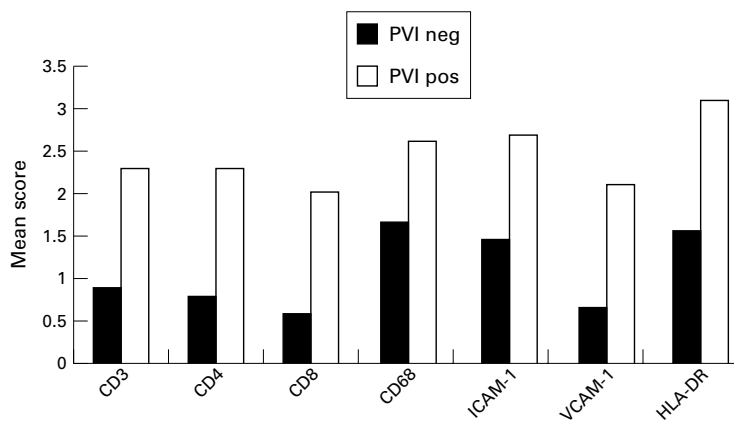


Figure 2 Infiltration by CD3+ T cells, CD4+ cells, CD8+ cells, and CD68+ macrophages, and expression of adhesion molecules ICAM-1 and VCAM-1 and the activation marker HLA-DR in muscle biopsy specimens in which perivascular infiltrates (PVI) were recorded on routine histology, compared with those in which such infiltrates were absent. Significantly higher mean semiquantitative scores for all the markers were documented when a perivascular infiltrate of more than three cell layers was present on routine histology ($p < 0.05$).

Table 3 Infiltration by CD3+ T cells, CD4+ cells, CD8+ cells, CD68+ macrophages, and expression of the adhesion molecules ICAM-1 and VCAM-1 and the activation antigen HLA-DR in muscle biopsy specimens of patients with RA and clinical signs of vasculitis without histological proof (RA+) and of patients with RA with vasculitis (RV) proved by the presence of fibrinoid necrosis of vessel walls or another histological gold standard for the diagnosis of vasculitis, according to the absence (PVI-) or presence (PVI+) of a perivascular infiltrate of more than three cell layers on routine histology. Values are the mean semiquantitative scores (standard error of the means)

	CD3	CD4	CD8	CD68	ICAM-1	VCAM-1	HLA-DR
RA+ PVI- (n=7)	1.3	1	0.8	2	1.5	0.9	2.1
RV PVI- (n=3)	1	1.3	1.3	3	3	1.3	2.3
All PVI- (n=10)	1.2 (0.1)	1.1 (0.1)	1 (0.2)	2.3 (0.3)	1.9 (0.3)	1 (0.2)	2.2 (0.2)
RA+ PVI+ (n=4)	2.5	2.2	2.3	2.7	2.7	2.2	3.5
RV PVI+ (n=11)	2.3	2.3	1.9	2.6	2.7	2.1	2.9
All PVI+ (n=15)	2.3* (0.2)	2.3* (0.2)	2* (0.3)	2.6 (0.2)	2.7 (0.3)	2.1* (0.2)	3.1* (0.2)

*p<0.05 (Student's *t* test comparing PVI- and PVI+).

Of all the patients with RA with clinical signs of vasculitis (RA+ and RV), the subgroup with perivascular infiltrates of more than three cell layers on routine histology had significantly higher scores ($p<0.05$) for infiltration by CD3+ T cells, CD4+ cells, and CD8+ cells as well as for expression of HLA-DR and VCAM-1 than the subgroup without such infiltrates (table 3). Moreover, the mean immunohistological scores for most markers were significantly higher in the RV subgroup without perivascular infiltrates than in the corresponding RA+ subgroup.

Discussion

The results presented here show that patients with RA with recent onset of extra-articular manifestations have significantly increased accumulation of activated inflammatory cells and increased expression of adhesion molecules in random quadriceps muscle biopsy specimens, even in the absence of conventional histological signs of vasculitis. The cell infiltrate and the expression of adhesion molecules and HLA-DR antigens in quadriceps muscle biopsy samples from patients with RA without any clinical suspicion of vasculitis are similar to the immunohistological features in biopsy samples from patients with OA.

Generally, rheumatoid vasculitis is defined histologically as the presence of an inflammatory infiltrate with destruction of the vessel wall. It is often not feasible to obtain biopsy specimens from the affected organ, which obviously may reduce the application of histological examination as a diagnostic test. In that case clinicians perform blind biopsies—for instance, of the quadriceps muscle. In these biopsy specimens vasculitis can be defined by the histology compared with the RA+ group.

Several clinical^{18,19} and experimental studies²⁰ suggest that rheumatoid vasculitis is caused by circulating immune complexes containing rheumatoid factor and autoantibodies such as anti-endothelial cell antibodies, forming deposits in vessel walls and triggering an inflammatory reaction, which may lead to endothelial cell injury. Apart from these humoral determinants, the morphological changes associated with the acute phase of vasculitis suggest a pathogenetic role for polymorphonuclear and mononuclear cells.²⁰

Adhesion molecules are upregulated by cytokines²¹ and have an important role in transendothelial migration of leucocytes.

The levels of circulating ICAM-1 and ICAM-3 are raised in patients with rheumatoid vasculitis compared with patients with RA without vasculitis.²²

The accumulation of activated cells and upregulated expression of adhesion molecules described here also reflect the pathophysiological events leading to vasculitis. It remains to be elucidated whether there is a difference, from a pathophysiological point of view, between symptomatic patients with histological proof according to the conventional vasculitis classification and those without. The higher degree of inflammation seen in the first group suggests that it might be only a matter of quantity. This could have therapeutic implications, as most clinicians rely on histology for the diagnosis of rheumatoid vasculitis and, consequently, their treatment strategies rely on histology as well.

Taken together, these results confirm and extend our previous study, suggesting changes on the vascular endothelium as well as perivascular cell infiltration in random quadriceps muscle biopsy specimens of patients with RA with clinical signs of vasculitis, regardless of histological proof according to the actual criteria for the diagnosis of vasculitis. The data indicate that the sensitivity of the examination of random muscle biopsy samples for the diagnosis of rheumatoid vasculitis can be increased by the use of new criteria. The ultimate value of these observations for differential diagnosis remains to be determined in larger clinical studies.

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